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Acetaminophen analog *N*-acetyl-*m*-aminophenol, but not its reactive metabolite, *N*-acetyl-*p*-benzoquinone imine induces CYP3A activity via inhibition of protein degradation

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ABSTRACT

Cytochrome P450 (CYP) 3A subfamily members are known to metabolize various types of drugs, highlighting the importance of understanding drug–drug interactions (DDI) depending on CYP3A induction or inhibition. While transcriptional regulation of CYP3A members is widely understood, post-translational regulation needs to be elucidated. We previously reported that acetaminophen (APAP) induces CYP3A activity via inhibition of protein degradation and proposed a novel DDI concept. *N*-Acetyl-*p*-benzoquinone imine (NAPQI), the reactive metabolite of APAP formed by CYP, is known to cause adverse events related to depletion of intracellular reduced glutathione (GSH). We aimed to inspect whether NAPQI rather than APAP itself could cause the inhibitory effects on protein degradation. We found that *N*-acetyl-L-cysteine, the precursor of GSH, and 1-aminobenzotriazole, a nonselective CYP inhibitor, had no effect on CYP3A1/23 protein levels affected by APAP. Thus, we used APAP analogs to test CYP3A1/23 mRNA levels, protein levels, and CYP3A activity. We found *N*-acetyl-*m*-aminophenol (AMAP), a regioisomer of APAP, has the same inhibitory effects of CYP3A1/23 protein degradation, while *p*-acetamidobenzoic acid (PAcBA), a carboxy-substituted form of APAP, shows no inhibitory effects. AMAP and PAcBA cannot be oxidized to quinone imine forms such as NAPQI, so the inhibitory effects could depend on the specific chemical structure of APAP.

1. Introduction

The cytochrome P450 (CYP) family of enzymes includes key enzymes responsible for metabolizing small-molecule drugs [1]. The effects of chemical compounds on metabolic activity need to be always considered in terms of drug–drug interactions (DDI). The CYP3A subfamily, the major human CYP, mainly contributes to the metabolism of pharmaceuticals [2,3]. The protein expression level of CYP3A4 in human liver microsomal fractions from several donors was reported to be highly correlated with the enzyme activity and mRNA expression levels [4]. Chemical compounds transcriptionally induce the CYP3A subfamily members via activation of nuclear receptors including pregnane X receptor (PXR) and constitutive androstane receptor (CAR) [5]. For instance, there is a good correlation between CYP3A4 activities and mRNA expression levels in response to typical inducers (e.g., rifampicin and dexamethasone) in human hepatocytes [6]. However, we recently reported that acetaminophen (APAP), an analgesic and antipyretic drug, induces functional rat CYP3A protein through inhibition of protein degradation [7].

CYP enzymes are localized in the endoplasmic reticulum (ER) membrane [8]. Both structurally altered by chemical compounds CYP3A and native CYP3A undergo ER-associated degradation, which involves the ubiquitin (Ub)-proteasome system [9]. We found that APAP significantly decreases the protein levels of poly-ubiquitinated CYP3A1/23 and glycoprotein 78 (gp78), the E3 ligase of CYP3A1/23, but why APAP decreases gp78 protein levels needs to be elucidated. Although APAP is considered a safe drug at therapeutic doses, overdoses often cause severe hepatotoxicity. APAP is metabolized successively by CYP1A2, 2E1, and 3A4 to the reactive metabolite, *N*-acetyl-*p*-benzoquinone imine (NAPQI). NAPQI is commonly conjugated by reduced glutathione (GSH) and

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detoxified, but excessive NAPQI depletes intracellular GSH levels, consequently leading to adverse events such as oxidative stress, protein nitration, and mitochondria dysfunction [10]. Therefore, the events induced by NAPQI are thought to be possible causes of reduction of gp78 protein levels.

N-Acetyl-*m*-aminophenol (AMAP), a meta regioisomer of APAP, has been used as a less toxic analog of APAP, in several mice *in vivo* and *in vitro* studies [11–14]. The difference of hepatotoxicity between APAP and AMAP can possibly be attributed to reactive metabolite target proteins properties in mice. On the other hand, in rat and human liver samples, AMAP is as toxic as APAP [13–15]. Xie et al. (2015) reported that AMAP forms mitochondrial protein adducts, causing mitochondria dysfunction and GSH levels reduction in primary human hepatocytes, which correlate with liver injury [14]. Although there is no studies on AMAP protein adducts in rat liver samples, AMAP mitochondrial protein adducts likely also cause gp78 protein levels reduction in rat hepatocytes. In addition, *N*-acetyl-*o*-aminophenol (AOAP), the ortho regioisomer of APAP, is reported to bind liver proteins when administered in excessive doses in laboratory animals [16], suggesting that these APAP regioisomers might have effects similar to APAP.

In the present study, we examined whether NAPQI and the lower GSH levels caused by APAP contribute to inhibit CYP3A1/23 protein degradation using 1-aminobenzotriazole (ABT), a nonselective CYP inhibitor, and *N*-acetyl-L-cysteine (NAC), a GSH precursor. Furthermore, we focused on APAP chemical structure and compared APAP analogs (Suppl Fig. 1) effects on CYP3A1/23 mRNA levels, protein levels, and CYP3A activity in rat hepatocyte spheroids. Besides APAP regioisomers, we tested also *p*-acetamidobenzoic acid (PAcBA), a carboxy-substituted form of APAP used as a component of the antiviral drug isoprinosine [17]. As PAcBA cannot be oxidized to the quinone form by CYP, it allowed us to determine whether the APAP quinone form contributes to CYP3A protein degradation inhibition by APAP.

2. Materials and methods

2.1. Materials

APAP, AMAP, AOAP, PAcBA, ABT, NAC, and anti-GAPDH antibody (G9545) were obtained from Sigma-Aldrich (St. Louis, MO, US). Anti mouse CYP3A1 antibody (sc-53246), anti rabbit gp78-2 antibody (sc-33541), and anti mouse ubiquitin antibody (sc-8017) were obtained from Santacruz Biotechnology (Dallas, Texas, US). Anti rabbit CYP3A1-antibody (BML-CR3310) were obtained from Enzo Life Sciences (Famingdale, NY, US). Glutathione Cell-Based Detection Kit (Blue Fluorescence) was purchased from Cayman chemical (Ann Arbor, MI, US) and P450-Glo™ CYP3A Assay with Luciferin-IPA from Promega (Fitchburg, WI, US).

2.2. Animals

Male Crj:CD (SD) rats (7 weeks old) were obtained from Charles River Laboratories (Kanagawa, Japan). The study was approved by the animal ethics committee of Hiroshima University.

2.3. Hepatocyte isolation and cell culture

Primary rat hepatocytes were isolated from SD rats using a collagenase perfusion method [18]. Cell culture was performed as previously described [7].

2.4. Immunoblotting analysis

Hepatocyte spheroids on micro-space cell culture plate (Elplasia™; Kuraray Co., Ltd.) were harvested as previously described [7]. Lysate supernatants were separated by 10% polyacrylamide gels and were transferred to PVDF membranes. CYP3A1, GAPDH, and gp78-2 were immunoblotted with primary rabbit polyclonal antibodies (BML-CR3310, G9545, and sc-33541 respectively). Goat anti-rabbit IgG (whole molecule)-Peroxidase (A9169) was used as a secondary antibody. 5% skim milk in Tween TBS (TBS-T) was used for blocking and for dilution of all primary and secondary antibodies. Densitometric quantitation was conducted using ImageQuant LAS 4000 mini (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK).

2.5. qRT-PCR analysis

Total RNA was extracted using SV Total RNA Isolation System (Promega, Fitchburg, WI). Quantitative real-time PCR was performed as previously described [7]. Expression levels of CYP3A1/23 mRNAs were normalized to β-2-microglobulin (β-2-m) levels. Gene expression was calculated using the standard curve method. Standard curve sample was prepared with QIAquick® Gel Extraction kit (QIAGEN, Hilden, Germany). The primers for CYP3A1/23 and β-2-m were designed as previously described [7].

2.6. CYP3A activity

CYP3A activity was evaluated with P450-Glo™ CYP3A Assay with Luciferin-IPA as previously described [7].

2.7. Intracellular GSH levels

Intracellular GSH levels were evaluated with Glutathione Cell-Based Detection Kit (Blue Fluorescence). Hepatocyte spheroids were washed twice with PBS (−) and harvested using a cell lysis buffer consisting of 50 mM HEPES, 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 10% Glycerol, and 1% Triton™ X-100. After centrifugation at 7000 rpm for 10 min at 4 °C, GSH levels in the supernatant were measured using Ensipire™ (PerkinElmer, Waltham, MA, US) accordingly to the kit protocol.

2.8. Synthesis of PAcBA sodium adducts

PAcBA was dissolved in ethanol, and was reacted with a half equivalent mole of Na_2CO_3. The reaction mixture was evaporated, and the residue was dissolved in acetone to completely dissolve the unreacted PAcBA. Finally, PacBA sodium adducts were separated by vacuum filtration.

2.9. Statistical analysis

Statistical significant differences were determined by one-way ANOVA with Tukey's, Student's, or Welch's *t*-test. A p value less than 0.05 was considered statistically significant.

3. Results

3.1. Intracellular GSH levels after exposure to APAP, ABT, and NAC

We confirmed that 10 mM APAP significantly decreased polyubiquitinated CYP3A1/23 and gp78 protein levels [7]. As APAP decreases intracellular GSH levels mediated by NAPQI, either NAPQI or the reduction of intracellular GSH levels could trigger these phenomena. APAP (10 mM) decreased intracellular GSH levels by 0.5 fold compared with control, but co-treatment with 500 μM ABT restored intracellular GSH levels by 0.8 fold compared with control. Moreover, co-treatment with 1 mM NAC increased intracellular

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